Midtlyng PJ (ed): Progress in Fish Vaccinology.
Dev Biol. Basel, Karger, 2005, vol 121, pp 143-150.

Development of a Cost-Effective Oral Vaccination Method Against Viral Disease in Fish

A.R. Companjen[1], D.E.A. Florack[2*], J.H.M.W. Bastiaans[1], C.I. Matos[2], D. Bosch[2], J.H.W.M. Rombout[1]*

Wageningen University and Research Centre:
[1] Department of Animal Sciences, Cell Biology and Immunology Group, and
[2] Plant Research International, Business Unit Bioscience, Wageningen, The Netherlands
* Arjen Companjen and Dion Florack contributed equally to this work

Abstract: Different vaccination methods have been applied to protect fish against the detrimental effects of various pathogens. Several studies have shown the potentials of oral vaccination. In theory oral vaccination is an effortless and stress-free method which can be applied at almost any age. In general, however, the vaccine has to be protected to avoid digestion, which results in high costs for application in aquaculture. In this paper we introduce a cost-effective oral vaccination strategy for viral diseases of fish. The vaccines discussed here include fusion proteins consisting of a gut adhesion molecule and a viral peptide expressed in plants. The adhesion molecule mediates binding to and uptake from the gut, whereas the viral peptide functions as vaccine antigen mediating the induction of a humoral immune response. The first pilot studies using a fusion of the gut adhesion molecule and well-characterised heterologous linear B- and T-cell viral epitopes, produced in potato tubers, showed a promising binding and subsequent uptake in the end gut of carp. The results further indicated that a specific humoral immune response was evoked.

Introduction

Since the beginning of mass vaccination in the fish industry, oral vaccination has been one of the methods applied [1]. This type of vaccination has been used since the mid-1960s to vaccinate humans and especially children on a mass scale against polio [2] and proved to be very successful. In fish especially, this type of vaccination is preferable since the vaccination via, for instance, injection is labour-intensive and stressful for fish, potentially resulting in significant losses. However, oral vaccination has some drawbacks: (i) the applied antigen (Ag) is often destroyed due to protease activity present in the intestinal tract; (ii) oral tolerance can be evoked and (iii) the Ag does not necessarily enter the gut mucosa and consequently

an immune response is not initiated. Destruction of the Ag in the gut can be avoided by encapsulation [3,4]. Uptake of the Ag may also be favoured by including a so-called mucosal carrier to direct the Ag into the gut mucosae.

Here we present a novel production system for oral vaccines using a plant expression system for application in aquaculture. Both in mouse and human models this system has been successfully tested [5-6,7 and references therein]. The recombinant vaccines discussed here are fusion proteins consisting of the non-toxic part of the *Escherichia coli* heat-labile enterotoxin B-subunit (LTB) as carrier molecule and viral peptides produced in potato tubers. As a model, a linear influenza T-cell epitope sequence [8] in conjunction with a linear B-cell parvo peptide [9] was used. In the experiments shown, potato suspensions containing either LTB or the LTB-influenza-parvo fusion protein were anally intubated in six- to nine-month old carp. Subsequent uptake of LTB or LTB-fusion protein (LTB-p) was monitored using specific antibodies against LTB and the parvo peptide. Humoral immune responses against LTB and parvo peptide were also assessed. We observed an uptake of LTB and parvo peptide in the end gut of carp at least six hours after anal administration. Antibody responses could also be detected, suggesting a response against LTB and parvo peptide.

Materials and Methods

Expression of LTB and LTB-influenza-parvo fusion protein in potato

Synthetic DNA sequences encoding LTB or LTB-influenza-parvo fusion protein were made and introduced into potato plants using the *Agrobacterium tumefaciens* transformation system [5]. The influenza and parvo epitopes used were as described [8,9] and expression was under control of the tuber-specific patatin promoter and nopaline synthase terminator. A schematic overview of gene constructs is represented in Figure 1.

Fish

Six- to nine-month-old carp (*Cyprinus carpio* L.) weighing around 75 g, were reared in our fish facility centre (De Haar vissen) in recirculating, filtered and UV-sterilised water at 23°C. The fish were fed with food pellets (Skretting/Nutreco, Putten, The Netherlands) at a daily ration of 2.5 % of their body weight. Twenty-four hours before intubation experiment the fish were not fed

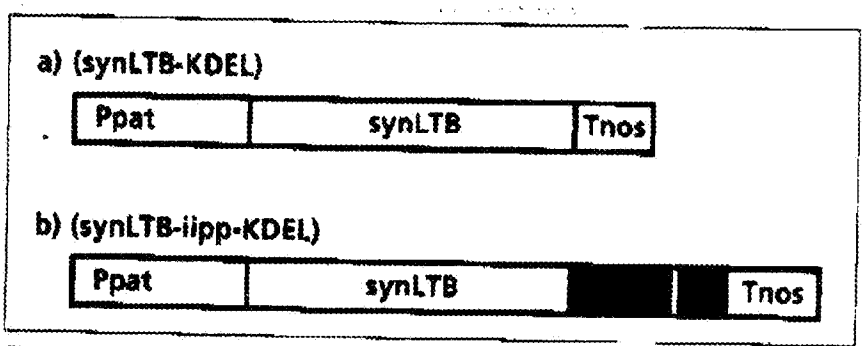

Fig. 1: Outline of the LTB or LTB-peptide constructs used in this study. a) construct containing the LTB gene followed by the sequence of the four consecutive amino acids: Lysine (K), Aspartic acid (D), Glutamic acid (E) and Leucine (L) (KDEL) (LTB); b) construct containing LTB followed by two influenza T-cell epitopes and two parvo B-cell epitopes and the KDEL sequence (LTB-p). For details see the Materials and Methods section. Ppat: patatin promoter; Tnos: termination sequence.

Uptake experiments

Potato tubers expressing either LTB or LTB-p were freeze-dried, ground and taken up in PBS (approximately 3 µg LTB/300 µl). Groups of three fish were anally intubated with 300 µl (= 3 µg LTB) potato suspension. Three and six hours post intubation the fish were killed by an overdose of Tricaine Methanesulphonate (TMS, Crescent Research Chemicals, AZ). The end gut was removed and fixed in 4 % paraformaldehyde (PFA, Merck Whitehouse Station, NJ) for 18 hours at 4°C. Subsequently the PFA fixed gut specimens were embedded in paraffin wax and stored at room temperature (RT) before use.

Immunisation experiments

Similar to the uptake experiments fish (n = 3/group) were intubated with 300 µl (= 3 µg LTB) potato suspension. One group of fish was killed three weeks post intubation and blood was collected (primary response). A second group of fish was boostered anally after eight weeks (secondary response) and killed two weeks post-booster, upon which blood samples were taken. The blood samples were allowed to clot for 18 hours at 4C. Subsequently, samples were centrifuged (10000 x g, 5 min. RT), serum was collected and stored at -80C before use.

Western blotting and immunodetection

Extracts of potatoes containing LTB or LTB-p were prepared as described [5], separated using precast 12.5 % SDS-PAGE gels and run under reduced conditions (BioRad, Hercules, CA). The proteins were blotted on to nitro-cellulose membranes (Schleicher and Schuell, Dassel, Germany) using an electroblot system (BioRad). The membranes were blocked with Tris buffered saline (TBS) containing 2 % low fat milk, 0.1 % bovine serum albumin and 0.1 % Tween-20 for one hour at room temperature (RT). Strips containing the separated potato proteins were subsequently incubated for one hour at RT with either a polyclonal antibody (pAb) recognising the LTB monomer (mcz12, kind gift of Dr. J. Langeveld), sera of carp intubated with LTB-p containing potato suspensions, or pre-immune serum. The blots were washed in TBS containing 0.1 % Tween-20 (TTBS, Merck) followed by incubation with horse radish peroxidase (HRP)-linked secondary antibodies or a monoclonal antibody recognising carp serum Ig (WCI-12, in the case of the serum incubated blots). Detection was with Lumi-Light Western blotting chemiluminescence substrate (Roche diagnostics, GmbH, Mannheim, Germany) using a Lumi-Imager (Boehringer Mannheim, Germany).

For the detection of accumulation of LTB or LTB-p in potato tubers, tubers of selected plants were cut and printed on nitro-cellulose paper by pressing. Blots were incubated as described above and probed with mAb recognising LTB pentamer (VD12 [5], or parvo peptide (3C9, Ingenaza, Madrid, Spain). Detection was with chemiluminescence as described.

ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 100 µl of 4 µg/ml anti-parvo peptide mAb (3C9, Ingenaza) followed by blocking with 0.5 % BSA (Roche, Mannheim, Germany) for two hours at RT. Subsequently Ag (synthetic parvo peptide, kind gift of Dr. J. Langeveld, 1 µg/ml) was added. Next, serum from carp, anally immunised with LTB-p or control serum, was added to some wells and serially diluted. Subsequently anti-parvo peptide specific serum antibodies were detected using biotin-linked mAb recognising carp serum Ig (WCI-12). Samples were diluted in PBS containing 0.5 % BSA and 0.1 % Tween 20 (Merck). Finally WCI-12 was detected using streptavidin-linked HRP (Sanquin, Amsterdam, The Netherlands, dilution: 1:5,000) and TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). The substrate was incubated for a maximum of 20 min, and subsequently the OD was measured at 450 nm. Anti-parvo titres were defined by the dilution of the sample at an OD of 0.1.

Immunohistochemistry

Sections of paraffin wax embedded in the end gut of carp, intubated with potato suspensions containing LTB or LTB-p, were cut using a microtome (Microm, MH 350, Heidelberg, Germany). The sections were mounted on poly-L lysine coated glass slides and rehydrated. Subsequently the sections were probed with a pAb recognising the LTB monomer (mcz12) or an mAb directed against the parvo peptide (3C9, Ingenaza). Next the sections were incubated with HRP linked anti-rabbit (BioRad, to detect anti-LTB pAb) or anti-mouse (BioRad, to detect anti-parvo mAb) Ig antibodies. Omission of primary antibody served as a negative control for the staining specificity. 3-amino-9-ethylcarbazole (AEC, Sigma, St. Louis, MO) was used as the chromogen.

RESULTS

Expression of fusion protein in potato tubers

In Figure 2 a tissue blot of selected tubers comprising the LTB-influenza-parvo fusion protein is shown. The left panel was incubated with LTB-specific monoclonal antibody and the right with parvo peptide specific monoclonal antibody, indicating accumulation of the fusion protein in the potato tuber.

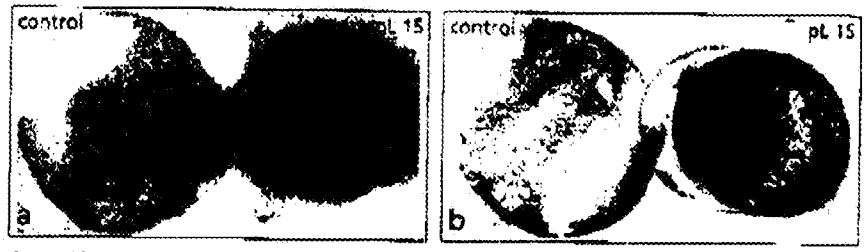

Fig. 2: LTB and parvo peptides are expressed in potato tubers transformed with LTB and LTB-p constructs. a) prints of potatoes transformed with empty constructs (control potato, left) or LTB-p constructs (pL15, right) probed with an mAb directed against LTB (VD12); b) prints of potatoes transformed with empty constructs (control potato, left) or LTB-p constructs (pL15, right) probed with an mAb directed against parvo peptide (3C9).

Potato derived LTB and LTB-p is taken up in carp gut mucosa

To monitor uptake of potato derived LTB or LTB-p in carp gut mucosa upon anal intubation, gut of carp treated with LTB or LTB-p containing potato suspensions was isolated. As shown in Figures 3b and c, LTB is clearly taken up in the gut mucosa six hours post intubation. LTB monomer could be detected in both brush border and supranuclear vacuoles. Macrophage-like cells also contained the LTB monomer, suggesting that the LTB can be transferred from the enterocytes into large macrophage-like cells in the mucosa (Fig. 3d). Fish treated with control potatoes show some background staining in the brush border (Fig. 3a) but the staining intensity is much lower when compared to the intensity observed in the LTB and LTB-p treated fish. Omission of primary antibody controls does not show any staining (data not shown). To assess whether the parvo peptide was taken up in gut mucosa, gut sections of carp were probed with the antibody specific for the parvo peptide. Clearly gut of fish intubated with potato-derived LTB-p show positive staining of both enterocytes and macrophage-like cells (Fig. 4), indicating that the parvo peptide is also taken up by the carp gut mucosa and suggesting that the fusion protein LTB-p is still intact. Gut sections of fish treated with control potato or potato containing LTB only did not display any staining for parvo as expected (data not shown). Uptake of LTB or LTB-linked parvo peptide could already be observed at three hours post intubation.

These data show that LTB as well as the LTB linked parvo peptide is readily taken up by both carp gut enterocytes and macrophage-like cells.

Fig. 3: Potato derived LTB and LTB-p is taken up in carp gut mucosa upon anal intubation. Sections of carp gut intubated with potato suspensions containing LTB or LTB-p and incubated for six hours, probed with pAb directed against LTB monomer (mcz12). a) gut of carp intubated with control potato suspensions, b) gut of carp intubated with potato suspensions containing LTB, c) gut of carp intubated with potato suspensions containing LTB-p, d) magnification of c) showing LTB positive macrophages and enterocytes (see arrowheads). Magnification: a, b and c: 100×; d: 400×.

Fig. 4:
Potato-derived LTB linked parvo peptide is taken up in carp gut mucosa upon anal intubation. Sections of carp gut intubated with potato suspensions containing LTB-p and incubated for six hours, probed with mAb directed against parvo peptide (3C9). Positive staining: see arrowheads. Magnification: 100×.

Humoral responses against potato-derived LTB and LTB-parvo peptide upon anal immunisation

Although it has been shown that potato-derived LTB as well as LTB linked parvo peptide was taken up in carp gut mucosa, it is still elusive whether an immune response is also evoked. Therefore, carp were anally immunised with potato suspensions containing LTB or LTB-p. Subsequently anti-LTB responses were measured by Western blot and anti-parvo peptide responses by parvo peptide-specific ELISA. Whereas blots incubated with carp pre-immune serum are negative, bands are visible on blots containing extracts of LTB transgene potatoes and probed with primary immune serum of LTB-p immunised fish (Fig. 5). Bands can also be seen

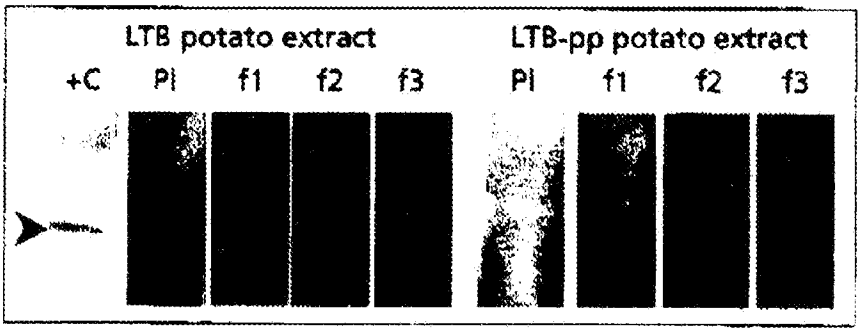

Fig. 5: Induction of LTB directed humoral immune responses. Extracts of potatoes containing LTB (LTB potato extract) or parvo peptide-linked LTB (LTB-p) were blotted and subsequently probed with an antibody recognising LTB monomer (mcz12, + c), pre-immune serum (PI) and primary immune serum of three different fish anally immunised with LTB-p (f1, 2 and 3). An arrow indicates the position of the LTB monomer.

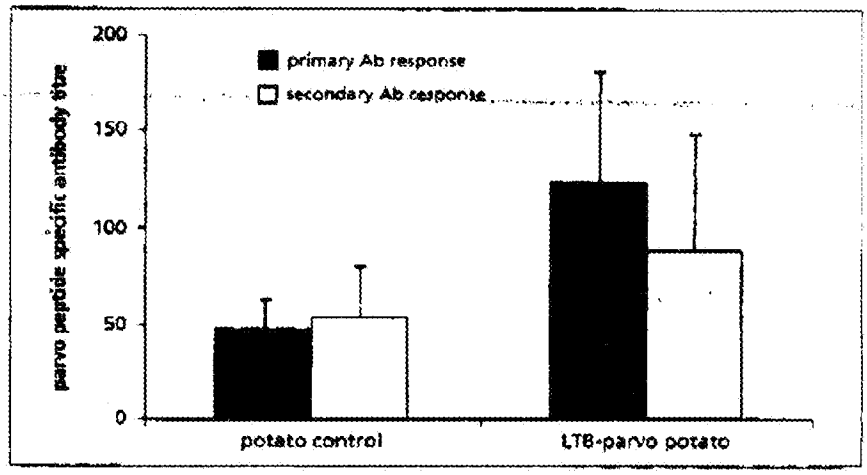

Fig 6: Induction of parvo peptide-directed immune responses. Parvo peptide-specific antibody responses were measured in primary and secondary immune serum of fish anally immunised with LTB-p. The parvo peptide-specific antibody responses were measured by ELISA. The mean antibody titre ± sd of three fish is shown.

on blots containing extracts of LTB-p transgenic potatoes. The immunoreactive molecules migrate approximately at the same level as the LTB-monomer (c. 12 kD). The bands visible on the LTB-p-containing blots are somewhat higher compared to the bands visible on the LTB containing blots. This might be explained by the difference in molecular weight of LTB compared to LTB-p, which also contains a stretch of peptides including a doubled parvo peptide. Anti-parvo responses in the serum of fish immunised with LTB-p were measured by ELISA. Figure 6 shows that the titres measured in the sera of fish in the LTB-p treated group is on average higher compared to the control group, although, significant results could not be obtained due to the relative small group of fish used in this pilot experiment.

Discussion

The use of edible vaccines and especially plant-derived edible vaccines for the protection against different diseases has repeatedly been reported [10 and references therein]. Here we use the plant expression system to produce oral vaccines to protect fish against viral diseases. As shown, the proteins of interest were readily expressed in the potato tubers. Subsequent, anal administration of potato suspensions containing LTB or parvo peptide linked to LTB resulted in binding and uptake of these proteins in the gut mucosa of carp. Clearly LTB and also the parvo peptide were localised from three hours post intubation in enterocytes and macrophage-like cells present in the end gut of carp. Parvo peptide was also taken up in fish treated with LTB-p-containing potatoes, whereas no staining was detected in the gut of fish intubated with control potatoes or potatoes with LTB only. Strikingly LTB and parvo peptide were located in macrophage-like cells. These LTB and parvo peptide-containing macrophages may be important in the induction of an immune response. Indeed carp anally intubated with LTB-p containing potato suspensions and incubated for three weeks (primary response), boostered after eight weeks and subsequently incubated for two weeks (secondary response) showed anti-LTP and anti-parvo peptide responses. Western blot analysis suggested that a LTB -directed antibody response was present in primary immune serum of carp intubated with potato suspensions containing LTB-p. Anti-parvo peptide specific antibodies could also be detected in both primary and secondary immune serum. This indicates that antibody responses are evoked against the anally-introduced LTB. Although the difference of the anti-parvo peptide responses between the control and the LTB-p intubated group was not significant because of the low number of fish tested, the anti-parvo titres of the LTB-p intubated group were consistently higher compared to the control group. Despite the low amount of LTB-p intubated (~3 µg LTB-p/fish), anti-LTB and anti-parvo peptide humoral responses were observed. This might suggest a specific accumulated uptake of the Ag facilitated by LTB, which binds to the cell surface ganglioside GM-1. The latter has recently been detected in carp gut [11] and hence is also most probably the receptor for our observed LTB-derived fusion protein uptake. In addition, LTB may have an adjuvant effect, which consequently boosts the amplitude of the humoral immune response even when very low concentrations of Ag are administered.

In conclusion it can be said that potato-derived LTB or LTB-linked parvo peptide is readily taken up in mucosal cells of carp gut and that LTB and parvo peptide-directed humoral immune responses might be evoked upon anal immunisation.

This is the first report on binding and uptake of LTB and LTB-derived vaccines in carp. These promising results suggest that oral vaccination of fish using edible vaccines produced in plants and based on the mucosal carrier molecule LTB, result in the initiation of an immune response. However, whether fish are indeed protected upon oral vaccination by plant-produced vaccines still has to be determined and experiments are currently in progress.

Acknowledgements

The authors wish to thank Ms. E. Kuenen-Fontijn for the immunohistochemistry studies, Ms. S. Heitinga for the Western blot studies and Dr. J. Langeveld for providing parvo peptide and antibodies 3C9 and m212. All experiments with genetically modified organisms are according to European guidelines 90/219/EC and 90/220/EC and were performed under the auspices of the Dutch Committee for Genetically Modified Organisms according to Dutch law and under licence GGO96-230 of the Dutch Ministry of Spatial Planning, Housing and the Environment. This project is funded by EU-grant QLK2-CT-2001-01288.

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Dr. A. Companjen, Cell Biology and Immunology Group, Wageningen University, NL-6709 PG Wageningen, The Netherlands.
E-mail: arjen.companjen@wur.nl